Interpreting the Regulatory Interplay in *E. coli* Metabolic Pathways

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Abstract. Many regulatory processes in the cell are based on the control of gene expression through the interaction of transcription factors. However, enzymatic regulation often overlays transcriptional regulation and even, in some metabolic pathways, enzymatic regulation prevails. The present study addresses the regulatory network of *Escherichia coli* and offers a global view of the regulation of its metabolic pathways. It identifies the regulatory mechanisms responsible for key metabolic activities and details the structures behind such mechanisms. This knowledge is considered of relevance to further studies on the bacteria's system and its industrial application, namely for understanding the signal cascades comprised in the responses to various environmental stresses.

1 Introduction

The analysis of biological networks aims at the understanding of metabolic capabilities of cells to adapt to, and to maintain growth under different external and internal conditions [1, 2]. A particularly challenging task is the inference of the regulatory interactions commanding the activity of metabolic pathways. Different mechanisms are recruited for regulation, either long-term regulation by changing the expression level of genes or short-term regulation by changing the activity of enzymes.

Gene expression is mostly controlled by transcription factors (TFs) that are proteins able to bind to gene promoter regions, inducing or repressing the initiation of

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gene transcription. In turn, the activity of enzymes, proteins that catalyze biochemical reactions, can be controlled by some effector molecules binding at the active or allosteric sites or by alteration of some environmental condition (e.g., pH or ionic strength).

In this work, we focus on the study of how transcriptional regulation couples with the regulation of the activity of metabolic pathways via enzymatic regulation, and how similar regulatory mechanisms are used across different metabolic pathways, i.e. the identification of regulatory circuits dominating certain pathway activities. Our goal is to obtain a global view of the regulatory interplay affecting the metabolism of the bacterium *Escherichia coli* (*E. coli*) K12, considering its common use as an industrial organism [3].

2 Methodology

The construction of a network integrating genome-scale transcriptional and enzymatic regulation requires information on gene encoding, gene regulation, gene-reaction associations, and enzymatic regulation. For this purpose, we can take previously validated metabolic and regulatory networks and/or retrieve information from publicly available repositories, and we need to perform the necessary data integration.

Here, we considered the gene-reaction associations from the genome-scale metabolic network of *E. coli* K12 (*i*AF1260) [4], and derived information on gene transcription regulation from the EcoCyc database [5]. Namely, TF-encoding genes that regulate the expression of metabolic genes (i.e. genes associated with a reaction from the *i*AF1260 model) and/or regulate other TF-encoding genes were included. Information on the control of enzyme activity was also obtained from EcoCyc and further associated with the respective enzyme-coding genes.

2.1 Network Analysis

Our network can be viewed as a graph with two types of nodes, genes and metabolites, and two types of edges: one that connects metabolites to genes that encode for enzymes they regulate; and another that connects pairs of genes that are linked by transcriptional regulation.

Using the representation provided by the Java Universal Network/Graph Framework (JUNG) (http://jung.sourceforge.net/), we analysed the properties of the graph. Specifically, the frequency of occurrence of different types of edges associated with metabolic genes determined the overlapping of transcriptional and enzymatic regulation, whereas regulatory patterns commonly described in literature [6, 7] provided deeper understanding on the interplays taking place on particular pathways (see details in Box 1).

To assess the importance of different regulatory motifs in particular metabolic pathways, we calculated the prevalence of motif types per pathway and the average number of genes in the pathway participating in such motifs.

Box 1

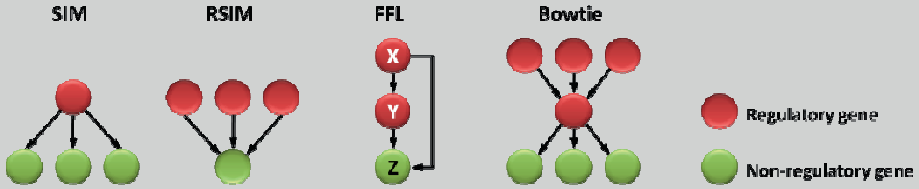
Network motifs

Single input module (SIM): two-gene pattern where a single regulatory gene is responsible for the regulation of more than one gene.

Reversed Single input module (SIM): a two-gene pattern where multiple regulatory genes govern the transcriptional regulation of one gene.

Feed-Forward Loop (FFL): three-gene pattern composed by two input regulatory genes, one of which regulates the other (X->Y), and both jointly regulate a target gene (Z).

Bowtie: three-gene pattern that includes a central node that is highly regulated and at the same time regulates multiple genes.



Specifically, for each pathway ***P*** and every motif type ***T***, we count the number of motifs that affect at least one gene in the pathway and denote it by ***Abs_Freq_T_in_P*** (absolute frequency of motif type ***T*** in pathway ***P***). We then calculate the relative frequency of motif type ***T*** in ***P***, i.e. the number of times the motif type ***T*** occurs in the pathway (affects at least one of its genes) divided by the number of times it occurs in the network, as:

$$\textit{Rel\_Freq\_T\_P} = \textit{Abs\_Freq\_T\_P} \,/\, (\#\ \textit{of motifs of type T in the network}) \quad (1)$$

Considering every gene G_P belonging to the pathway ***P***, the relative frequency of G_P in motif type ***T*** denotes how many genes in the pathway, in average, are expected to be included in at least one occurrence of such motif:

$$\textit{Rel\_Freq\_}G_P\textit{\_T}: \textit{Count all\_genes\_in\_ motif\_T\_P} \,/\, \#\ \textit{of genes\_in\_P} \quad (2)$$

3 Results and Discussion

3.1 Types of Regulation

As illustrated in Fig. 1, transcriptional regulation as the only form of regulation is dominant in the *E. coli* metabolism (37% of genes are regulated at least by one TF), although co-regulation (i.e., the combination of transcriptional and enzymatic regulation) and enzymatic regulation are also present (16% and 13% of genes, respectively). This evidences that metabolic activities do not necessarily correlate proportionally with the gene expression levels of the corresponding enzymes, but are also dependent, in almost 30% of the cases, on immediate control over the enzymes.

The high percentage of unknown regulation (37%) can be explained by the insufficiency of knowledge (it has not yet been possible to assign a function to

approximately one third of the proteins identified in *E.coli* and many details are still missing from its biochemical characterisation [8]) and the non-inclusion of other regulatory mechanisms in this study (e.g. posttranslational modifications and ribosome-mediated transcriptional attenuations).

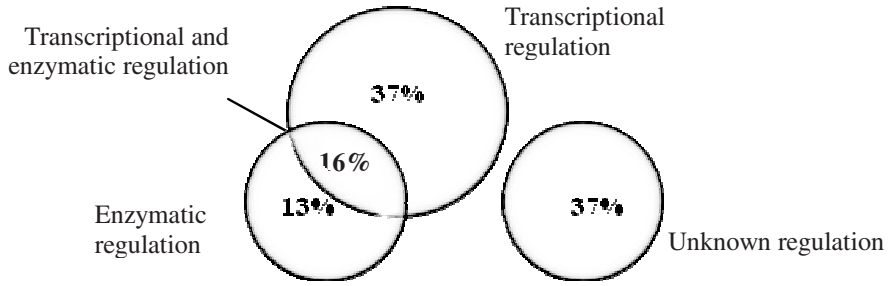


Fig. 1 The different types of regulation in the *E. coli* network.

Details on the regulation *per* pathway (Table 1) enables the generation of hypotheses about the mechanisms coordinating key metabolic processes. The transcriptional regulation is dominant in pathways like "Nitrogen Metabolism", "Citric Acid Cycle", "Inorganic Ion Transport and Metabolism" and other transport pathways. These pathways have in common the fact that their response to environmental inputs, such as availability of nitrogen and carbon sources, requires long-term regulation, i.e. modifications at gene expression level, to be able to adjust their activities accordingly. On the other hand, the existence of co-regulation in amino acids biosynthesis pathways, like "Glutamate Metabolism", "Tyrosine, Tryptophan, and Phenylalanine Metabolism" and "Arginine and Proline Metabolism", can be explained by the need to ensure both a longer-term regulation and the fine-tuning of metabolic activities coupled with the rapid response to over-accumulation of end-products.

3.2 Regulatory Motifs

Many metabolic pathways are dependent on the activity of transcriptional regulators that are often organized as regulatory structures or motifs acting as specific functional modules (Table 2). The association of certain structures with particular metabolic activities can be hypothesized as a consequence of specific information processing. Besides the biological relevance of these regulatory structures to modulate the activity of numerous biochemical functions, it is important to recognize that often motif overlap reveals that some genes respond to multiple regulatory mechanisms.

SIM and RSIM motifs are simple regulatory structures required to coordinate the activity of multiple genes at the metabolic level. While SIMs represent a set of genes that are controlled by a single TF (i.e. one-to-many), RSIMs display a single gene being controlled by multiple regulators (i.e. many-to-one). That is, many metabolic activities are dependent on the regulation of a single regulator (SIMs) or

Table 1 Effect of different types of regulation in *E. coli* pathways. The gradient of colours illustrates the incidence of a given type of regulation in a pathway (increasing incidence ranges from black to red).

Pathway	Number of Genes	Transcriptional regulation	Metabolic regulation	Transcriptional and metabolic regulation	Unknown regulation
Alanine and Aspartate Metabolism	11	27%	9%	27%	36%
Alternate Carbon Metabolism	159	48%	6%	21%	26%
Anaplerotic Reactions	10	20%	40%	20%	20%
Arginine and Proline Metabolism	40	23%	18%	40%	20%
Cell Envelope Biosynthesis	53	17%	17%	11%	55%
Citric Acid Cycle	18	83%	0%	11%	6%
Cofactor and Prosthetic Group Biosynthesis	136	20%	16%	7%	57%
Cysteine Metabolism	20	35%	15%	20%	30%
Folate Metabolism	4	25%	50%	25%	0%
Glutamate Metabolism	11	0%	9%	64%	27%
Glycerophospholipid Metabolism	20	15%	35%	0%	50%
Glycine and Serine Metabolism	15	27%	7%	40%	27%
Glycolysis/Gluconeogenesis	32	31%	13%	28%	28%
Glyoxylate Metabolism	4	25%	0%	25%	50%
Histidine Metabolism	9	0%	22%	11%	67%
Information Transfer	167	56%	0%	0%	44%
Inorganic Ion Transport and Metabolism	67	76%	1%	0%	22%
Lipopolysaccharide Biosynthesis / Recycling	46	7%	9%	2%	83%
Membrane Lipid Metabolism	14	50%	14%	7%	29%
Methionine Metabolism	13	31%	0%	23%	46%
Methylglyoxal Metabolism	10	0%	20%	10%	70%
Murein Biosynthesis	10	20%	0%	0%	80%
Murein Recycling	34	29%	6%	0%	65%
Nitrogen Metabolism	15	87%	0%	7%	7%
Nucleotide Salvage Pathway	57	19%	26%	19%	35%
Oxidative Phosphorylation	77	65%	8%	5%	22%
Pentose Phosphate Pathway	13	15%	31%	23%	31%
Purine and Pyrimidine Biosynthesis	22	41%	18%	27%	14%
Pyruvate Metabolism	20	50%	15%	15%	20%
Threonine and Lysine Metabolism	19	11%	37%	21%	32%
Transport, Inner Membrane	216	72%	0%	1%	26%
Transport, Outer Membrane	21	81%	0%	0%	19%
Transport, Outer Membrane Porin	4	75%	0%	0%	25%
tRNA Charging	24	0%	17%	4%	79%
Tyrosine, Tryptophan, and Phenylalanine Metabolism	24	29%	4%	29%	38%
Unassigned	23	39%	4%	13%	43%
Valine, Leucine, and Isoleucine Metabolism	17	76%	0%	18%	6%

are subjected to regulation from various regulators (RSIMs). Pathways like “Alternate Carbon Metabolism” and “Transport, Inner Membrane” are examples of metabolic functions that depend on this immediate form of regulation to respond to external *stimuli* (e.g. nutrient carbon sources).

Bowtie structures can be interpreted as the coupling of SIM and RSIM motifs through a single central element, suggesting that these structures share a similar conceptual and architectural design. The capacity of central nodes to admit variability of input information (i.e. regulation from other genetic elements), confers high flexibility and robustness to the system, while supporting the modulation of multiple pathways simultaneously [9]. Pathways like “Transport, Inner Membrane” and “Information Transfer”, which support inherently complex information exchange processes, are in need of these regulatory structures to guarantee the adequate propagation of the information throughout the network.

Table 2 Statistical evaluation of the occurrence of regulatory motifs *per* pathway. Abbreviations: *T_P*, relative frequency of a motif type *T* in pathway *P*; *Gp_T*, frequency of genes from the pathway *P* (*Gp*) involved in motif type *T* (see section *Network Analysis* for details on the metrics used).

Pathway	FFL			SIM			RSIM			BowTie		
	#	*T_P*	*Gp_T*	#	*T_P*	*Gp_T*	#	*T_P*	*Gp_T*	#	*T_P*	*Gp_T*
Alanine and Aspartate Metabolism	3	0,00	0,27	6	0,05	1,00	4	0,01	0,36	2	0,10	0,18
Alternate Carbon Metabolism	144	0,13	0,91	60	0,46	1,74	82	0,17	0,52	9	0,45	0,21
Anaplerotic Reactions	6	0,01	0,60	7	0,05	1,70	3	0,01	0,30	-	-	-
Arginine and Proline Metabolism	10	0,01	0,25	15	0,12	1,33	12	0,02	0,30	3	0,15	0,15
Cell Envelope Biosynthesis	-	-	-	9	0,07	0,38	4	0,01	0,08	1	0,05	0,02
Citric Acid Cycle	61	0,06	3,39	17	0,13	4,11	17	0,03	0,94	6	0,30	0,89
Cofactor and Prosthetic Group Biosynthesis	45	0,04	0,33	27	0,21	0,63	23	0,05	0,17	5	0,25	0,10
Cysteine Metabolism	6	0,01	0,30	6	0,05	0,85	4	0,01	0,20	1	0,05	0,05
Folate Metabolism	2	0,00	0,50	5	0,04	1,25	1	0,00	0,25	-	-	-
Glutamate Metabolism	41	0,04	3,73	13	0,10	3,18	6	0,01	0,55	5	0,25	1,18
Glycerophospholipid Metabolism	7	0,01	0,35	8	0,06	0,45	2	0,00	0,10	1	0,05	0,05
Glycine and Serine Metabolism	7	0,01	0,47	12	0,09	1,40	6	0,01	0,40	2	0,10	0,13
Glycolysis/Gluconeogenesis	15	0,01	0,47	10	0,08	1,06	8	0,02	0,25	3	0,15	0,09
Glyoxylate Metabolism	-	-	-	1	0,01	0,50	-	-	-	-	-	-
Histidine Metabolism	-	-	-	1	0,01	0,11	-	-	-	-	-	-
Information Transfer	96	0,09	0,57		0,00		57	0,11	0,34	16	0,80	0,36
Inorganic Ion Transport and Metabolism	38	0,04	0,57	28	0,22	1,54	26	0,05	0,39	5	0,25	0,39
Lipopolysaccharide Biosynthesis / Recycling	-	-	-	4	0,03	0,13	2	0,00	0,04	1	0,05	0,04
Membrane Lipid Metabolism	2	0,00	0,14	7	0,05	1,21	7	0,01	0,50	1	0,05	0,14
Methionine Metabolism	3	0,00	0,23	6	0,05	1,00	6	0,01	0,46	1	0,05	0,08
Methylglyoxal Metabolism	1	0,00	0,10	2	0,02	0,20	1	0,00	0,10	-	-	-
Murein Biosynthesis	-	-	-	1	0,01	0,10	-	-	-	-	-	-
Murein Recycling	2	0,00	0,06	8	0,06	0,59	6	0,01	0,18	-	-	-
Nitrogen Metabolism	49	0,05	3,27	15	0,12	5,53	11	0,02	0,73	4	0,20	1,53
Nucleotide Salvage Pathway	16	0,01	0,28	18	0,14	0,82	12	0,02	0,21	2	0,10	0,14
Oxidative Phosphorylation	218	0,20	2,83	24	0,18	3,35	52	0,10	0,68	8	0,40	0,65
Pentose Phosphate Pathway	3	0,00	0,23	8	0,06	0,77	3	0,01	0,23	2	0,10	0,15
Purine and Pyrimidine Biosynthesis	4	0,00	0,18	8	0,06	1,27	8	0,02	0,36	3	0,15	0,23
Pyruvate Metabolism	42	0,04	2,10	17	0,13	2,50	13	0,03	0,65	3	0,15	0,50
Threonine and Lysine Metabolism	5	0,00	0,26	11	0,08	0,63	4	0,01	0,21	3	0,15	0,16
Transport, Inner Membrane	229	0,21	1,06	86	0,66	1,93	111	0,22	0,51	18	0,90	0,39
Transport, Outer Membrane	12	0,01	0,57	18	0,14	1,76	7	0,01	0,33	2	0,10	0,33
Transport, Outer Membrane Porin	8	0,01	2,00	9	0,07	3,50	2	0,00	0,50	1	0,05	0,25
tRNA Charging	-	-	-	1	0,01	0,04	-	-	-	-	-	-
Tyrosine, Tryptophan, and Phenylalanine Metabolism	2	0,00	0,08	7	0,05	0,79	4	0,01	0,17	-	-	-
Unassigned	17	0,02	0,74	16	0,12	1,26	7	0,01	0,30	4	0,20	0,35
Valine, Leucine, and Isoleucine Metabolism	15	0,01	0,88	10	0,08	1,76	7	0,01	0,41	2	0,10	0,18

FFL motifs represent again a simple form of regulation where the activities of two TF-coding genes regulate the expression of the target gene both directly and indirectly. These motifs are likely to occur when a rapid response to an external signal is required, such as shifts in carbon sources or availability of oxygen [10]. For that reason, pathways like "Oxidative Phosphorylation", "Transport, Inner Membrane" and "Alternate Carbon Metabolism" present higher abundance of these motifs. Also, most of the genes associated with these pathways are controlled by this regulatory circuit, allowing a rapid functional switch in response to a *stimulus*. Next, we detail some peculiar pathways in terms of regulatory mechanisms.

3.2.1 Folate Metabolism: A Tightly Short-Term Regulated Pathway

The folate metabolism is central to many cellular processes in *E. coli,* ranging from nucleotide and amino acid biosynthesis to the production of the starting amino acid residue in protein synthesis, i.e. N-formylmethionyl-tRNA(f).

The enzyme-coding genes *pur*U and *folD*, core of this metabolism, are only regulated enzymatically by the amino acids glycine and methionine, and coenzyme formyltetrahydrofolate (Fig. 2a). This fact is explained by the need to balance the pools of tetrahydrofolate and one-carbon tetrahydrofolate metabolites to maintain the synthesis of glycine and methionine.

The metabolic gene *met*F, which is associated to the reduction of folate coenzymes, is the only one to be regulated transcriptionally, specifically by a SIM structure based on the TF-coding gene *met*J, which is known to repress the expression of genes involved in biosynthesis and transport of methionine.

3.2.2 Citric Acid Cycle and Nitrogen Metabolism: Two Pathways Depending on Long Term Regulation

Both the "Citric Acid Cycle" and "Nitrogen Metabolism" relate to respiratory catalytic pathways. The "Citric Acid Cycle" is a catabolic pathway of aerobic respiration [11]. The "Nitrogen Metabolism" comprising the periplasmic nitrate reductases (Nap) and nitrite reductases (Nrf), encoded by the napFDAGHBC and *nrf*ABCD operons respectively, supports cell growth via nitrate/nitrite respiration under anaerobic conditions [12, 13].

Unlike the previous example, these pathways do not have much incidence of enzymatic regulation. The isocitrate dehydrogenase is the only enzyme in the "Citric Acid Cycle" with enzymatic regulation and is subjected to allosteric control by oxaloacetate, i.e. the end product of the "Citric Acid Cycle", and glyoxylate, i.e. an anabolic intermediary that is present under specific physiological conditions (Fig. 2b). This modulation enables cells to make rapid shifts between "Citric Acid Cycle" and “Glyoxylate Metabolism” pathways and thus, adjust cellular growth to different carbon sources.

Most enzymes in both pathways are controlled via transcriptional regulation, in particular via FFL and SIM structures. For example, the *suc*AB operon in the "Citric Acid Cycle" and the *nap*ABCDGH and *nrf*ABCD operons in "Nitrogen Metabolism" are regulated by multiple TFs through FFLs (Figs. 2b and 2c). This

suggests that changes in the environmental conditions are counteracted by the combined action of multiple regulators, such as CRP, IHF, ArcA, FNR or Fis.

3.2.3 Glutamate Metabolism: A Pathway Depending on Co-regulation

The "Glutamate metabolism" was highlighted for discussion due to the significant co-occurrence of transcriptional and enzymatic regulation. This is a very important pathway, because glutamate is a major constituent of the proteins in *E. coli* and it is a major nitrogen donor for other biosynthesis activities.

One possible example of the complexity of this pathway is the activity of the two glutamate decarboxylases that participate in the acid resistance system controlling responses to low pH [14]. The coding genes *gad*A and *gad*B are regulated by multiple TFs, and FFLs are the common regulatory structure (Fig. 2d). The enzymes are also affected by various metabolic regulators. Intermediates of the "Citric Acid Cycle", such as fumarate and succinate, act upon these enzymes to inhibit their activity when pH is back normal. These combined actions enable the cell to respond quickly to pH perturbations.

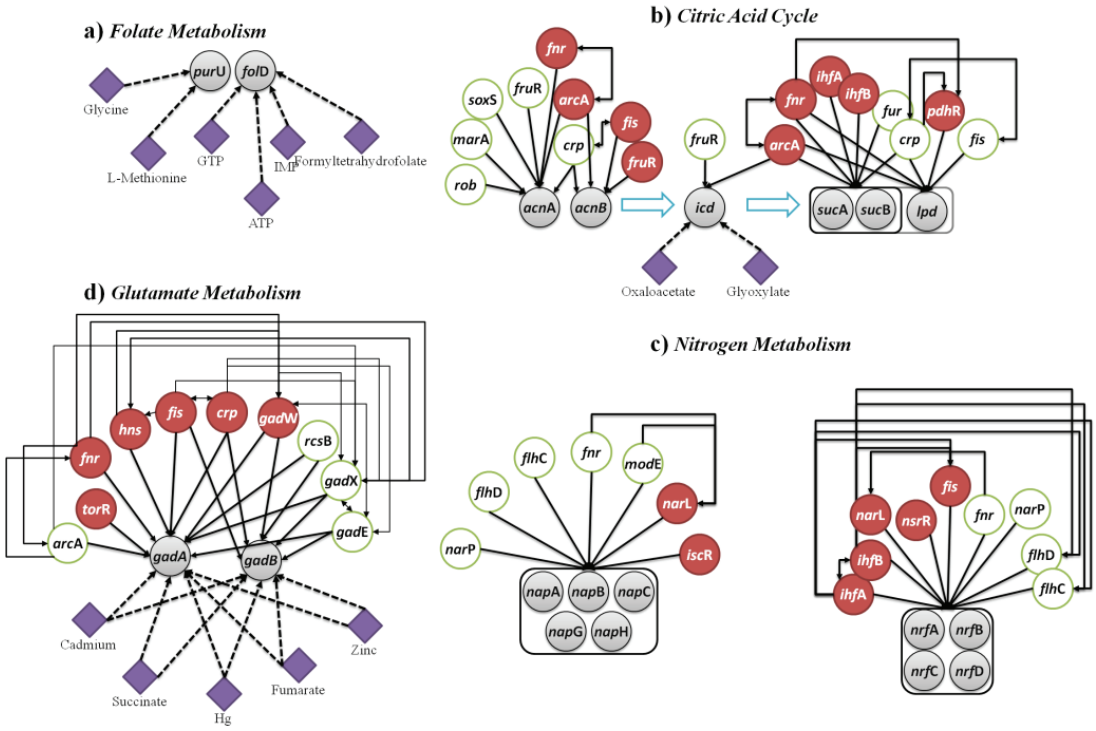


Fig. 2 Simplified representations of four pathways exhibiting particular regulatory motifs.

4 Conclusions

Considering that TFs and metabolic regulators have similar functional purposes, i.e. to ultimately regulate the activity of enzymatic reactions, the integrated analysis of their activities provides a new perspective over the capacity of *E. coli* to modulate metabolic pathways. TF-based regulation is meant to perform system

adaption whereas enzymatic regulation is chosen when a rapid shift in a given metabolic activity is needed. For example, the shift from aerobic respiration to anaerobic respiration requires gene expression adjustments as the cells have to adapt their entire metabolism to a new environment. In turn, enzymatic regulators are needed to balance certain metabolic pools and thus maintaining the concentration of end products within acceptable ranges.

Information processing is supported by various structures of regulation, capable of responding to one or more environmental/internal inputs. Each structure has a unique way to process information (it may receive multiple inputs and/or it may affect multiple gene targets) and its relevance over a pathway is given by the number of affected genes that belong to the pathway. This view is meant to discriminate between pathways that are heavily regulated and those where TF regulation is scarce. Additionally, the study has shown that certain regulatory structures are characteristic of a subset of pathways. In particular, the ability to accept a wide range of inputs and to convey information through a single node affecting several functional elements is seen in pathways where the transference of information is critical.

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